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Abstract

The fps/fes proto-oncogene encodes a 92 kDa protein tyrosine kinase. To understand the physiological function of Fps we have generated a knockout mouse line that lacks Fps expression. Fps-knockout female mice produce litters that gain weight more slowly than wildtype mice, and develop breast tumors more quickly than wildtype mice. These data suggest that Fps participates in regulating mammary gland development and tumorigenesis. To address these hypotheses we are examining the biological and biochemical function(s) of Fps in the mammary gland.

Over the past year we have established cell culture conditions that allow for the isolation and expansion of highly purified mammary epithelial cells from wildtype and fps-null mice. These cells will be used as an in vitro model to examine the function of Fps in the mammary gland. Analysis of whole mammary tissue has revealed that Fps (and Fer) interact with protein components of the E-cadherin based adherens junction complex. Specifically, E-cadherin, β-catenin and p120-catenin interact with Fps and Fer in the mammary gland during the lactation stage. This data is supported by confocal microscopy which demonstrates co-localization between Fps and E-cadherin. Despite the presence of fps and Fer in the adherens junction there is no detectable tyrosine phosphorylation of any of the components. Future experiments will address the precise role that Fps and Fer play in regulating the function of the adherens junction.

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Introduction:

The fps/fes proto-oncogene encodes a 92 kDa protein tyrosine kinase. To understand the biological function of the Fps kinase we have generated a knockout mouse (fps-null) model that lacks the Fps protein. During the initial analysis of this mouse line two important observations were made. First, litters reared by fps-null mice gained weight significantly more slowly than wildtype mice, and was determined to be dependent upon the genotype of the mother. Second, using a breast cancer mouse model, breast tumors developed earlier in the fps-null genetic background compared to the wildtype background. These observations suggest that Fps has an important role in the normal development of the mammary gland and possibly as a suppressor of mammary tumor development. The overall purpose of this research proposal is designed to elucidate the biological and biochemical function(s) of Fps in the mammary gland by 1) identifying the morphological abnormalities associated with the loss of Fps expression, 2) identifying the signaling pathways in which Fps participates and 3) determining important interacting protein molecules and substrates that Fps regulates. It is anticipated that this research will show that Fps regulates one or more stages of mammary gland development, possibly the lactation stage, and may function in pathways or cell processes that inhibit tumor initiation and/or progression.

Body:

Objectives #1,2,3 of this proposal were initiated in year 1 and are either completed or partially completed.

Objective #1: Determine the pattern and level of expression of the fps gene in the mammary gland.
#1A. Generate constructs for anti-sense RNA expression of Fps and an epithelial marker.
#1B. Perform RNA *in situ* hybridization analysis on whole mammary glands and histological sections
#1C. Northern blot analysis on RNA from different stages of pregnancy and lactation to obtain a quantitative expression profile of the *fps* gene.

Although the methods described in the original proposal were not used, the objectives were nonetheless **completed**, and provided equally informative data. If the information gained from the unfinished experiments is viewed as being critical to a publication then they can be accomplished in a relatively short period of time. However, at this time we consider the information gained from such experiments to be somewhat redundant to what has already been produced and therefore unnecessary.

Objective #2: Perform mammary gland morphological analyses.

#2A. Whole-mount mammary gland staining analysis.
#2B. Histological analysis of mammary gland sections.

Wholemount analysis showed that there is no noticeable difference in the overall branching pattern of the epithelial ducts in mammary glands from wild-type and fps-null mice. Because Fps is most active during lactation we had planned to use a computer program to quantitate the ductal branching and alveolar structures of the different genotypes. However, the thickness of the wholemount glands and the density of the ducts and alveoli made this approach not practical. Instead, we decided it would be best to focus on H&E stained sections of lactating glands. Originally we thought the epithelial cells lining the alveoli structures in *fps-null* glands had a distorted shape, with the apical surface protruding into the ductal space. This is still our basic contention. However, we have not yet conclusively identified this phenotype. This is in part due to the heterogeneity between tissue samples and within each tissue itself. Currently, we are deciding on an appropriate mouse mammary gland researcher to establish a collaboration and then obtain an accurate assessment of the possible

differences between *wild-type* and *fps-null* tissue. We have not performed a quantitative assessment of the number of secretory alveoli structures within each gland but it is still in our future plans.

Objective #3: Generation of mammary epithelial cell lines from transgenic mice with different *fps* genetic backgrounds for *in vitro* studies.

#3A. Isolation of primary epithelial cells and growth in culture.
#3B and C. Immortalization of primary cells and characterization of cell lines to confirm their epithelial lineage.

Previously it was reported that we had adopted a method for primary epithelial isolation and growth in culture. Via this method we obtain a highly enriched epithelial cell population from a crude mammary gland cell suspension. In brief, mammary tissue is minced with scissors and digested with collagenase A and hyaluronidase for two to three hours. The suspension is then filtered to remove large undigested pieces and washed extensively to remove the digestive enzymes. The remaining cells/cell clumps are plated in a collagen gel and allowed to proliferate in serum free conditions supplemented with insulin and EGF. These conditions promote epithelial growth and inhibit fibroblast growth (**Figure 1**). After two to three weeks of growth the collagen gel is digested away and the cells are washed and replated on collagen coated culture dishes. At this point the cells are greater than 95% pure (**Figure 2**), based on the cobblestone morphology and a lack of flatter elongated "fibroblastic" cells. Thus, the ability to grow epithelial cells has been acquired. However, it has been very difficult to work with the cells in subsequent steps. The problems include: 1) when grown in collagen gel the cells are often in large clumps (up to 500 cells or more), 2) difficulty in getting an even distribution of cells over the surface of the culture dish because of the large clumps, 3) a lack of proliferation of the cells once taken out of the collagen gel, 4) extreme sensitivity of the cells to apoptosis.

With the mammary epithelial cells in hand the next step was to generate immortalized cells. Several attempts have been made to immortalize both wild-type and fps-null epithelial cells with a retroviral based system. So far this has been attempted with retrovirus encoding Sv40 LT, si p53, Polyoma LT or Sv40 LT tsA58 (temperature sensitive form). Unfortunately, I am unable to report any immortalization events. Each of these systems allows for antibiotic selection to remove untransfected cells. After selection, only fibroblast (based on morphology) remained. This indicates that infectious virus was produced but they either did not infect the epithelial cells or the cells were not proliferating sufficiently to allow for integration and transgene expression. In addition to the retroviral based experiments I have also started to use a lentivirus system to generate immortalized cells. The advantage of this system is in its ability to infect non-dividing cells. Initial attempts with a lentivirus expressing GFP have been successful based on the GFP expression of a small fraction of cells (data not shown). The next step will be to generate a lentiviral plasmid that expresses an appropriate immortalization agent. We must also take into account that the normal function of these cells must not be disrupted during their immortalization. The reason for this is that in order to study Fps function the cells will need to retain the ability to differentiate in the presence of lactogenic hormones. Concurrent with these experiments we have decided that in the absence of a cell line to study Fps function much of the proposed experiments can be performed using primary epithelial cells. We have just recently begun to use the primary cells on an experimental basis but do not yet have any reportable outcomes. Together, I think these approaches will allow us to study the function of Fps in epithelial cells in an *in vitro* setting.

Objective #4: Assessment of the role of Fps in mammary epithelial cell signaling *in vitro*.

Initially the completion of this objective depended on the success of Objective #3. However, in the absence of mammary epithelial cells we have used the mammary gland itself as the starting material to investigate the function(s) of Fps. This approach has its limitations. For example, starvation/growth factor stimulation experiments are not able to be performed. However, it is still useful and has provided some important data regarding the function of Fps in the mammary gland. Although we have yet to identify any Fps substrates using this approach we have identified some interesting interacting proteins that are implicated in cell adhesion and tumorigenesis.

#4A. Perform stimulations with growth factors known to act on epithelial cells.

- not initiated yet.

#4B. Investigate signaling pathways in which Fps is activated and assess its contribution to the activation of downstream signaling pathways.

Because Fps is highly expressed and active during lactation most of the experiments have focused on this stage of development to study the function of Fps. Additionally, because our original data suggested that *fps-null* mice displayed a lactation defect and also an earlier tumor onset compared to *wild-type* mice, we surmised that Fps was likely involved in a number of signaling pathways including milk protein expression, secretion, trafficking, cell adhesion and cell-cell interactions. Initially we examined some known and possible regulators of milk production and secretion/trafficking/membrane recycling. One of two methods was used for these experiments; 1) immunoprecipitations (IPs) followed by western analysis with a PY99 antibody or 2) standard western analysis with control and phosphospecific antibodies.

In both wt and null tissue we have looked at:

- STAT5 levels and phosphorylation, with no difference between the genotypes (data not shown).
- Pacsin 2 levels and phosphorylation, with no difference between the genotypes (data not shown).
- Pacsin 3 levels and phosphorylation, with no difference between the genotypes (data not shown).
- N-WASP levels and phosphorylation, with no difference between the genotypes (data not shown).
- Dynamin 2 levels and phosphorylation, with no difference between the genotypes (data not shown).
- Cortactin levels and phosphorylation, with no difference between the genotypes (data not shown).
- Synaptojanin levels and phosphorylation, with no difference between the genotypes (data not shown).
- NSF levels and phosphorylation, with no difference between the genotypes (data not shown).

Some of these results are preliminary and additional experiments are needed to show conclusively that no differences exist between the genotypes.

We have also examined levels and phosphorylation status of proteins involved in proliferation and survival. No differences in ERK, p38 or Akt signaling could be detected by western blot analysis using control or phosphospecific antibodies (data not shown).

With respect to cell adhesion, we tested p190 RhoGAP, Integrin β1, Integrin α2, FAK (p120) and p130 Cas. Similarly, no differences in expression levels or phosphorylation status could be detected (data not shown).

Unfortunately, analysis of the aforementioned proteins did not reveal any potential Fps substrates. Part of the difficulty lies with the fact that we do not know on which signaling pathway to focus our attention. However, other candidate substrates will be tested in the future. This depends in part on identifying logical targets and the availability of quality antibodies.

#4C. Assess the role of Fps in cell-cell adhesion.

To address this objective we focused on the E-cadherin based adherens junction. Initial experiments focused on determining if there was an interaction between Fps and members of the adherens junction complex. Basically, IPs were performed with antibodies that recognize Fps or components of the adherens junction and then immunoblotted with an antibody against the protein in question. First, we examined whether Fps and E-cadherin interacted in lactating glands from *wt* and *fps-null* mice. In Fps/Fer IPs, an intense band corresponding to E-cadherin was detected in the *wt* sample while a very faint band was observed in the null sample (**Figure 3**). This result indicates that E-cadherin does indeed interact with Fps in the lactating mammary gland. The weaker signal detected in the absence of Fps suggests that Fer is also a component of the adherens junction during lactation. Next, we addressed whether this interaction was specific to the lactation stage. Wild-type mammary tissue from various stages of pregnancy and lactation were harvested and subjected to a similar analysis. It appears that in late pregnancy Fps and E-cadherin begin to associate and then during lactation this interaction is highly upregulated (**Figure 4**). Then we examined the interaction of Fps (and Fer) with β-catenin and p120 catenin. In IPs of β-catenin and p120 catenin both Fps and Fer were detected in *wt* tissue and only Fer was present in *fps-null* tissue (**Figure 5**). Similarly in Fps/Fer IPs, E-cadherin, β-catenin and p120 catenin were detected in both *wt* and *fps-null* samples, with a weaker signal for each in the *fps-null* lanes.

Having established that Fps (and Fer) are components of the adherens junction we then determined the phosphotyrosine status of each adherens junction protein. Each component was IPed and analyzed by western blotting using the PY99 antibody (**Figure 6**). Despite the presence of Fps and Fer neither E-cadherin, β-catenin nor p120 catenin displayed any tyrosine phosphorylation. The phosphotyrosine status has not been determined for other adherens junction proteins like α-catenin and PTP1B but experiments are planned to do so in the near future. Also, it is still not known if any of the adherens junction proteins can act as a substrate for Fps or Fer. In the very near future this question will be addressed through immunoprecipitation experiments followed by *in vitro* kinase assays. This should provide some important information about the function of Fps/Fer in the adherens junction. We will also employ IP and western blotting techniques to examine the stoichiometry of the adherens junction components in *wt* and *fps-null* glands.

The biochemical data demonstrating an E-cadherin/Fps interaction was supplemented with confocal analysis which showed that a small fraction of the total Fps protein co-localized with E-cadherin at the lateral and basal membrane surfaces of the mammary epithelial cells (**Figure 7**). Experimental procedures for this aspect of the project were described in Annual Report #1. In brief, lactating tissue was harvested, fixed overnight in 10% formalin in PBS and then given to a departmental technician for sectioning. Following a standard immunohistochemical protocol, sections were incubated with antibodies to Fps and E-cadherin and then the appropriate Alexa Fluor conjugated secondary antibodies. The sections were covered with a coverslip and then viewed using a Leica TCS SP2 scanning confocal microscope through a 100X objective. Multiple samples still need to be tested to determine if this is a feature common to the *fps-null* mammary gland.

Objective #5. Perform cDNA microarray analysis of whole mammary glands and epithelial cell lines with the different *fps* genetic backgrounds.

- not yet initiated.

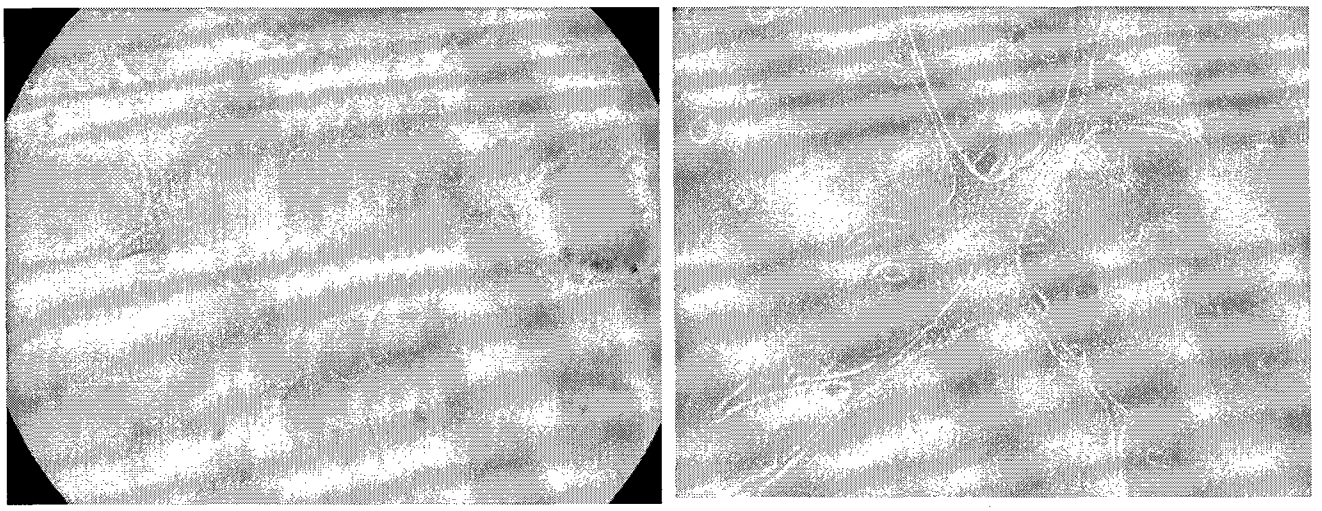

Figure 1. Mammary gland epithelial cells from mid-pregnant mice grown in collagen gel. After 2-3 weeks masses of epithelial cells begin to grow and form 3-dimensional ductal structures. The left photo is a low magnification view, and the right photo is a more magnified image of an individual structure. Both *wild-type* and *fps-null* cells produce similar structures.

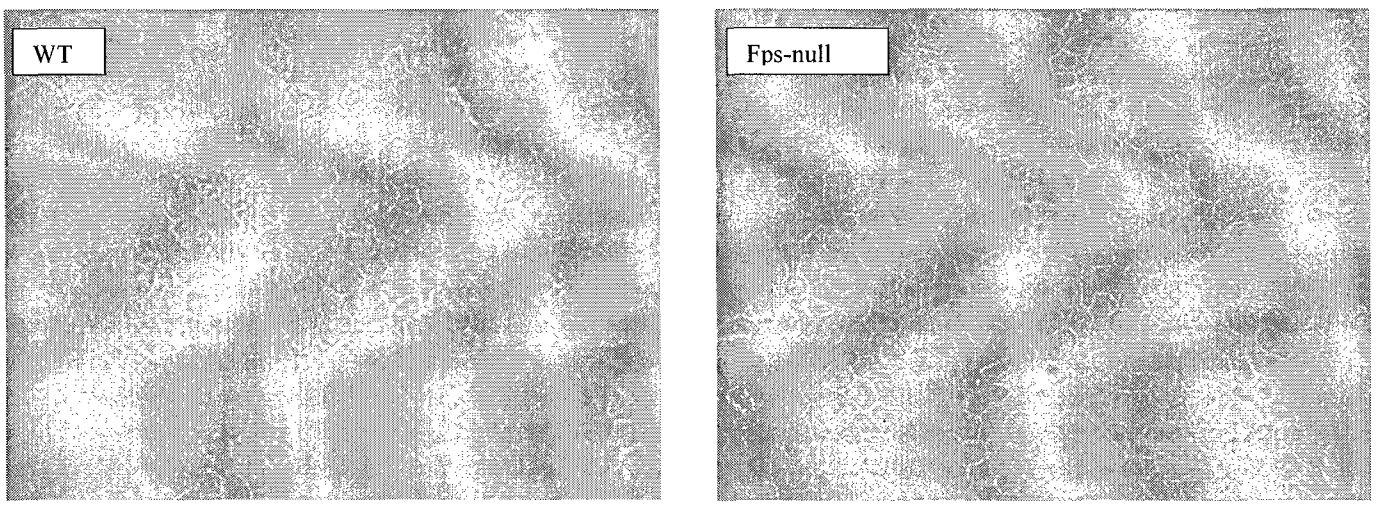

Figure 2. Mammary gland epithelial cells growing on a culture plate. After growth in a collagen gel for 3 weeks the cell clumps were recovered by digestion of the collagen and then allowed to adhere to a collagen coated plate and continue growing. The characteristic cobblestone morphology can be seen in both wt and fps-null cells. There does not appear to be any obvious differences in the size or shape of the cells between the genotypes.

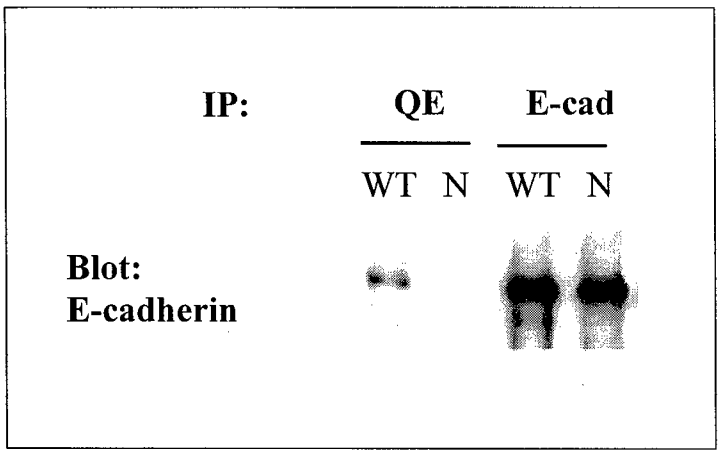

Figure 3. Interaction of E-cadherin with Fps in the lactating mammary gland. Mammary glands from *wt* and *fps-null* lactating mice were analyzed by immunoprecipitation with the FpsQE antibody that recognizes both Fps and Fer or the E-cadherin antibody. Western analysis with the E-cadherin antibody showed that Fps interacts with E-cadherin. A less intense band can also be seen in the QE IP of the *fps-null* sample after a longer exposure.

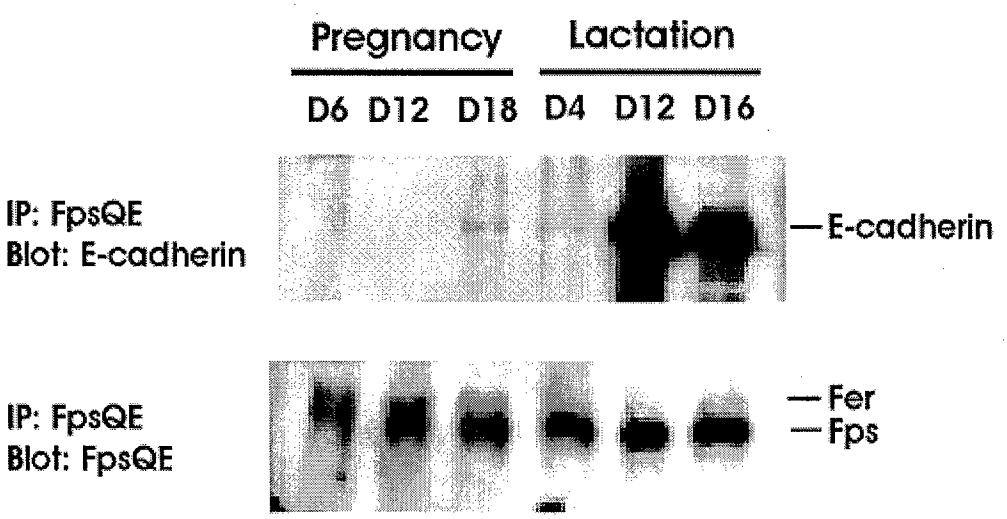

Figure 4. Interaction of E-cadherin and Fps during late pregnancy and lactation. *Wild-type* mammary glands from different stages of pregnancy and lactation were analyzed by immunoprecipitation with the FpsQE antibody. Western blot analysis with the E-cadherin antibody showed that Fps and E-cadherin interact predominantly during stages of lactation and to a lesser degree during late pregnancy. The lower panel shows that similar amounts of Fps/Fer were IPed from the different stages of mammary development. Please note that FpsQE IPs from lactation day 4 usually have an amount of E-cadherin that is similar to the later stages of lactation.

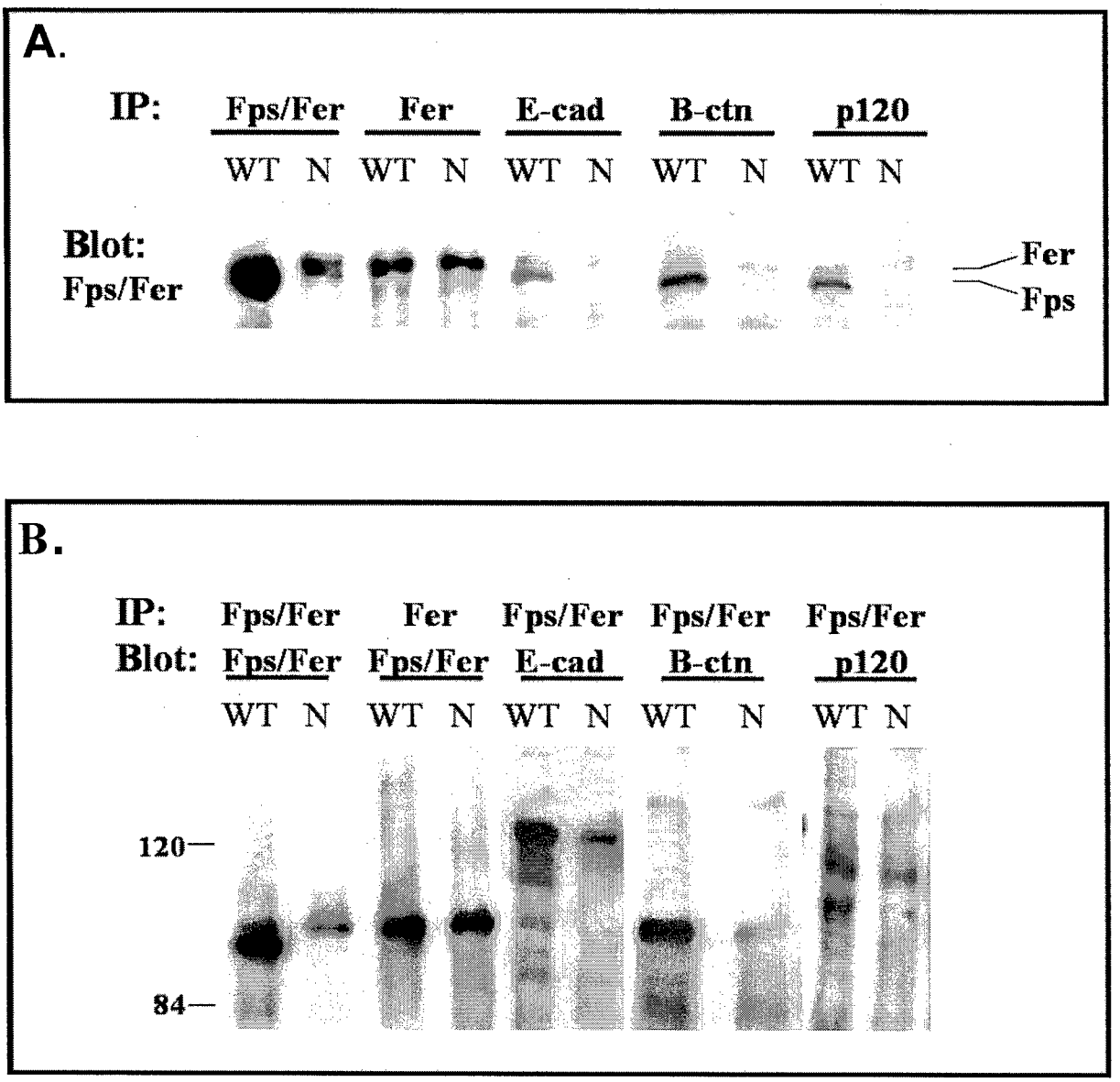

Figure 5. Interaction of Fps (and Fer) with components of the adherens junction in lactating mammary glands. Lysates from *wild-type (*WT*)* and *Fps-null (*N*)* mammary glands were subjected to immunoprecipitation (IP) analysis using antibodies that recognize Fps/Fes, Fer, E-cadherin, β-catenin or p120 catenin. In (A), the indicated IPs were blotted with an antibody that detects both Fps and Fer. In *wild-type* samples both Fps and Fer are present in IPs of Fps/Fer, E-cadherin, β-catenin and p120catenin, whereas only Fer is present in IPs from *Fps-null* tissue. In (B), IPs using the α-Fps/Fer or α̃-Fer antibody were probed with antibodies to Fps/Fer, E-cadherin, β-catenin or p120catenin. In the *wild-type* samples a signal corresponding to the appropriate protein was detected. A slightly weaker signal was present in *fps-null* samples. Together, these results indicate that both Fps and Fer are components of the adherens junction complex in lactating mammary glands.

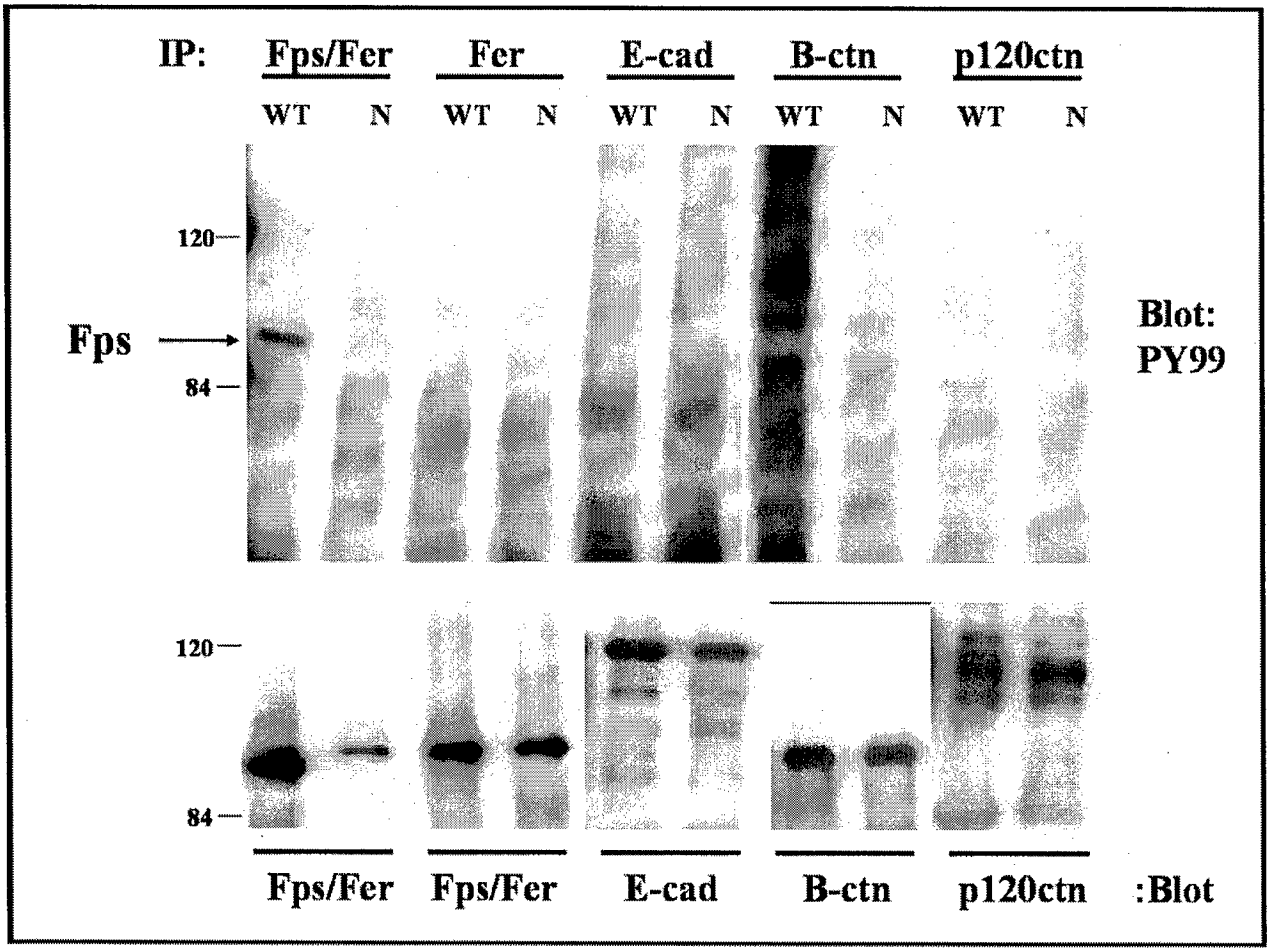

Figure 6. Phosphorylation of adherens junction proteins in the lactating mammary gland. Mammary gland lysates from *wild-type (WT)* and *Fps-null* (N) mice were immunoprecipitated with the specified antibody and immunoblotted with an α-phosphotyrosine antibody. The upper panel indicates that Fps is tyrosine phosphorylated in Fps/Fer IPs from *wild-type* tissue. However no phosphoproteins corresponding in size to E-cadherin, β-catenin or p120catenin were apparent in Fps/Fes IPs. Furthermore, there was no apparent difference in the phosphotyrosine status of E-cadherin, β-catenin or p120 catenin, or associated proteins, in their respective IPs. The lower panel demonstrates that equal amounts of these proteins were IPed between genotypes for each analysis.

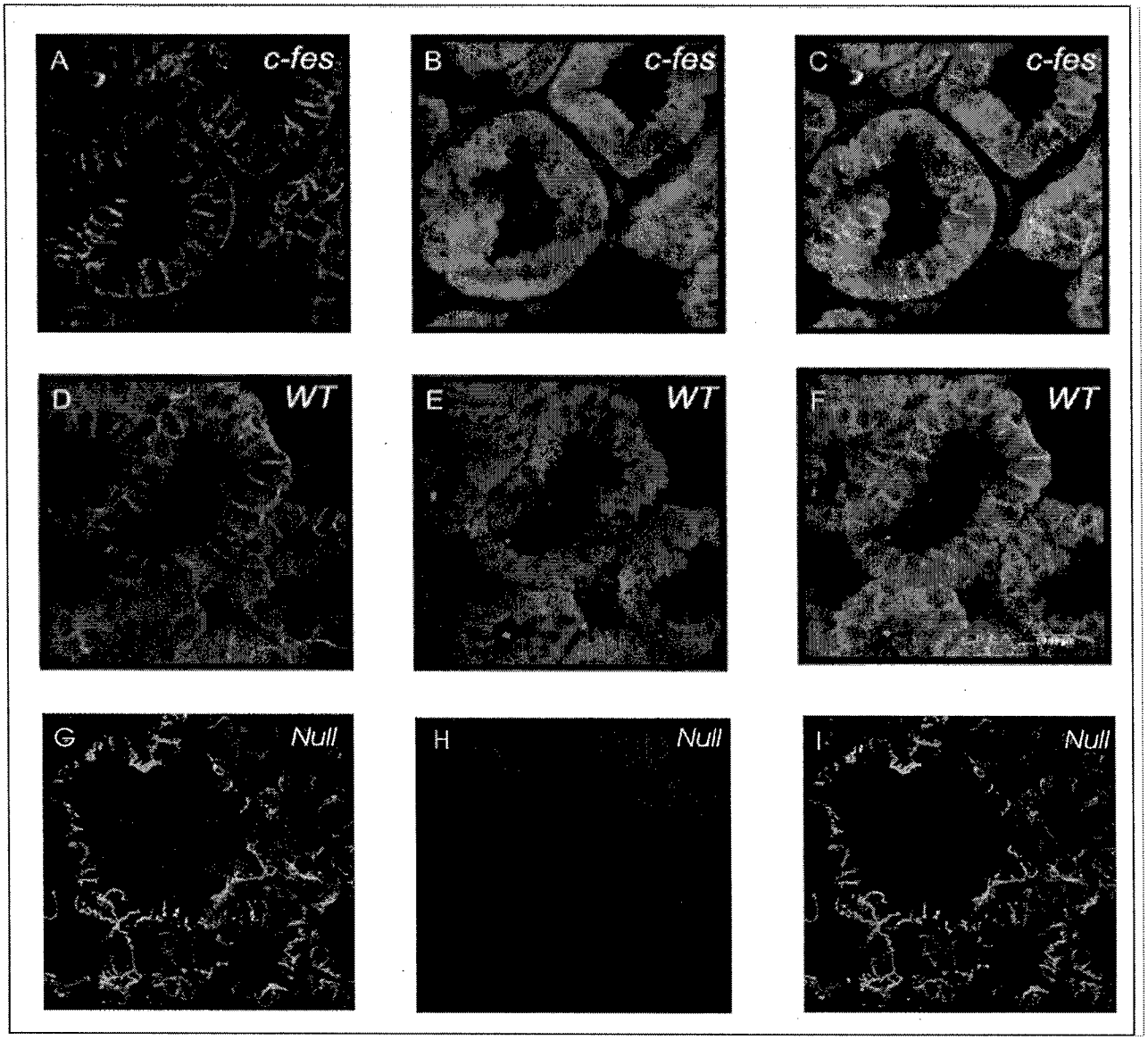

Figure 7. Cellular localization of Fps and its co-localization with E-cadherin in the lactating mammary gland. Breast sections from *c-fes* transgenic mice tissue-specifically over-expressing human Fps/Fes (A, B and C), *wild type* mice (D, E and F) and *fps-null* mice (G, H, and I) were incubated with α-Fps/Fer and α-E-cadherin antibodies. E-cadherin (A, D and G) was visualized in green by incubating with Alexa 488- conjugated goat α-mouse IgG $F(ab)_2$ fragments. Fps and Fer (B, E and H) were visualized in red using Alexa 633- conjugated goat α-rabbit IgG $F(ab)_2$ fragments. Adjacent green and red images were merged in C, F and I. Together these results show that Fps is expressed predominantly in epithelial cells and a fraction of this expression co-localizes with E-cadherin. In the absence of Fps expression it appears that the E-cadherin staining and adherens junctions are less organized.

Key Research Accomplishments:

- We have established a quality method to isolate and grow mammary epithelial cells.
- Fps and Fer are components of the E-cadherin based adherens junction, primarily during lactation.
- Directly or indirectly, Fps and Fer interact with E-cadherin, β-catenin and p120 catenin.
- From confocal analysis, E-cadherin staining in fps-null glands is different from that in wt glands.

Reportable Outcomes:

1. Poster presentation at the 43rd Annual Meeting of the American Society of Cell Biology 2003. Title: **Characterization of Fps Tyrosine Kinase Function in the Mouse Mammary Gland during Lactation**. Authors: Peter Truesdell, Ralph Zirngibl and Peter Greer.

2. Poster presentation at the 44th Annual Meeting of the American Society of Cell Biology 2004. Title: **The Fps/Fes Protein Tyrosine Kinase is a Component of the Adherens Junction in the Murine Mammary Gland during Lactation**. Authors: Peter Truesdell, Sarah Francis and Peter Greer.

3. Poster presentation at the Semi-annual Meeting of the Era of Hope 2005. Title: **The Fps/Fes Tyrosine kinase is Upregulated in the Mouse Mammary Gland during Lactation and is a Component of the E-cadherin based Adherens Junction**. Authors: Peter Truesdell and Peter Greer.

Conclusions:

Although the research has not progressed entirely as planned over the past year we have made some important observations that help explain the possible role(s) of Fps in mammary development and tumorigenesis. Despite the fact that an actual substrate of Fps has not been identified, at least the proteins that have been examined can be eliminated from the list of potential candidate substrates. I have dedicated several months of research to this goal without success, so it has not been for a lack of effort.

We have finally achieved the ability to grow highly pure epithelial cells from both wt and fps-null glands. An immortalized cell line has yet to be produced but attempts are still being made. Hopefully the lentiviral system will make this goal more attainable.

The most important discovery that has been made in the past year is that Fps and Fer are components of the E-cadherin based adherens junction. Specifically, Fps and Fer are in a complex with E-cadherin, β-catenin and p120 catenin. The result was supported by confocal analysis showing a colocalization of Fps and E-cadherin. It appears that these interactions may be specific to the lactation stage. Other stages of mammary development have not been thoroughly tested and it will be important to determine if there is a constitutive association between Fps/Fer and the adherens junction. It will be important to determine what role Fps plays in regulating AJ function. This would then establish a relationship between Fps, and possibly Fer, with proteins that have a direct link to the initiation and progression of breast cancer.

Training Accomplishments:

I have gained a great deal of valuable knowledge and experience over the past year in isolating and growing primary mammary epithelial cells, and maintaining them in culture. It has been a difficult struggle to get to this stage of the project but I view it as an important learning experience that will be beneficial to future endeavors involving mammary epithelial cells. I plan to rely on this expertise when I employ a more complex culturing method to grow the cells in a 3-dimensional matrigel and then use lactogenic hormones to induce differentiation that will hopefully involve the Fps kinase.